The role of paraoxonase (PON1) in the detoxication of organophosphates and its human polymorphism

L.G. Costa [a],*, W.F. Li [a], R.J. Richter [b], D.M. Shih [c], A. Lusis [c], C.E. Furlong [b]

[a] *Department of Environmental Health, 4225 Roosevelt # 100, University of Washington, Seattle, WA 98105, USA*
[b] *Department of Medicine, University of Washington, Seattle, WA, USA*
[c] *Department of Medicine, UCLA, Los Angeles, CA, USA*

Abstract

In human populations, serum paraoxonase (PON1) exhibits a substrate dependent polymorphism. The Arg_{192} isoform hydrolyzes paraoxon rapidly but diazoxon, soman and especially sarin slowly. On the other hand, the Gln_{192} isoform hydrolyzes paraoxon slowly, but diazoxon, soman and sarin more rapidly than the Arg_{192} isoform. Our experiments with a mouse model system have convincingly shown that PON1 plays a major role in the detoxication of organophosphate (OP) compounds processed through the P450/PON1 pathway. Recent studies have also shown that PON1 plays an important role in the metabolism of oxidized lipid compounds. Currently, there is an effort underway to identify genes and polymorphisms that play an important role in 'environmental susceptibility'. The PON1 polymorphism has been cited as a prime example of such a genetic polymorphism. The advent of the polymerase chain reaction (PCR) for DNA amplification with improvements, modifications and automation has provided a very convenient way to do individual genotyping. It is tempting to set up large scale PCR analyses of populations to determine individuals at risk for environmental exposures affected by the PON1 polymorphism. In fact, a number of such studies have already been carried out in examining the relationship of the

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* Corresponding author. Tel.: + 1-206-5432831; fax: + 1-206-6854696.
E-mail address: lgcosta@u.washington.edu (L.G. Costa)

PON1 polymorphism to vascular disease. We advocate the use of a high throughput two-dimensional enzyme assay that provides both PON1 genotype and phenotype (PON1 status). The high level of variation of gene expression within each genetic class in humans, together with our animal model studies indicate that it is very important to determine PON1 status as opposed to PON1 genotype alone. Experiments in rats and mice have shown that injection of PON1 purified from rabbit serum by the i.v., i.p. or i.m. route, significantly increases PON1 activities in rodents' plasma. Under these conditions, the acute toxicity (assessed by the degree of acetylcholinesterase inhibition) of paraoxon and chlorpyrifos oxon is significantly decreased, compared to control animals. Protection is maximal when PON1 is administered before the OPs, but still occurs when PON1 is utilized as a post-exposure treatment. Furthermore, protection by PON1 is also provided toward the parent compound chlorpyrifos. *Pon1*-knockout mice display a much greater sensitivity to chlorpyrifos oxon toxicity than wild mice. However, the acute toxicity of guthion, which is not a substrate for PON1, does not differ between knockout and wild mice. These observations underline the importance of considering both genetic variability of enzyme isoform as well as enzyme level (PON1 status) and the developmental time course of appearance of PON1 in developing risk assessment models. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

1. The PON1 polymorphism

Serum paraoxonase received its name from the ability to hydrolyze paraoxon, the toxic metabolite of the insecticide parathion. Norman Aldridge [1] proposed the designation of A-esterase for this enzyme. For many years, however, there was a controversy as to whether paraoxonase and the serum A-esterase were one or two different polypeptides. Activity stains of gels [2] and expression studies [3] clearly demonstrated that paraoxonase (now designated PON1) and the A-esterase were indeed one and the same protein. Population enzyme activity distributions where the rates of phenylacetate hydrolysis are plotted against the rates of paraoxon hydrolysis for individuals, are also in accord with these observations [4]. Krisch [5], Geldmacher von Mallinckrodt et al. [6] and Playfer et al. [7] observed that the activity of PON1 was multimodally distributed in human populations. Following these observations, a number of laboratories developed different assays with which to investigate this interesting polymorphism [8].

Finally, in 1991 human [9,10] and rabbit [9] PON1s were purified. The availability of pure enzyme allowed for the cloning of the rabbit PON1 cDNA, which in turn was used to isolate human PON1 cDNA [11]. The availability of the cDNA sequences allowed for the determination of the molecular basis of the PON1 activity polymorphism [12,13]. Human PON1 is 355 amino acids long and retains all but the methionine residue of its secretion signal sequence [11], whereas rabbit PON1 is a 359-amino acid protein that also retains its signal sequence and is 85% identical to the human protein. More recently, sequences for the mouse [3,14,15] and dog [16] PON1 cDNAs have been reported. Comparison of the PON1 amino

acid sequences shows a high degree of conservation, particularly of specific domains.

Based on the polymorphism of PON1 in human populations and the known role of A-esterase in detoxifying the active metabolites of several organophosphates (OPs) (Fig. 1), it has been inferred for some time that the expression of this enzyme plays an important role in determining susceptibility to OPs [17]. Yet, direct proof of this hypothesis has been obtained only in the past few years by a number of animal studies that are summarized herein.

2. Development of a mouse model for in vivo detoxication studies

The oxygen analogs of a number of OPs (e.g. paraoxon, chlorpyrifos oxon, diazinon oxon) are hydrolyzed by the serum A-esterase, paraoxonase or PON1, which appears to play a central role in their detoxication and in their toxicity [18]. While paraoxonase activity can be detected at low levels in most tissues, very high levels are present in rat liver and plasma, with plasma accounting for more than 50% of 'whole body' paraoxonase activity [19].

Initial evidence for a role of PON1 in OP toxicity was provided by the observation that injection of an 'A-esterase concentrate' from rabbit serum into rats, could protect them from the acute toxicity of paraoxon given by i.v. injection [20]. Further correlative evidence came from studies that showed animals with low PON1 levels were more sensitive to specific OP compounds than animals with high enzyme levels. For example, birds, which have very low to undetectable PON1 activity, are more sensitive than various mammals to the acute toxicity of paraoxon, diazinon oxon and pirimiphos oxon [21,22]. Further, rabbits, which have a sevenfold higher serum PON1 activity than rats, are fourfold more resistant to the acute toxicity of paraoxon [23].

A series of studies carried out in the past several years have added evidence that serum PON1 activity plays a most relevant role in determining the sensitivity to
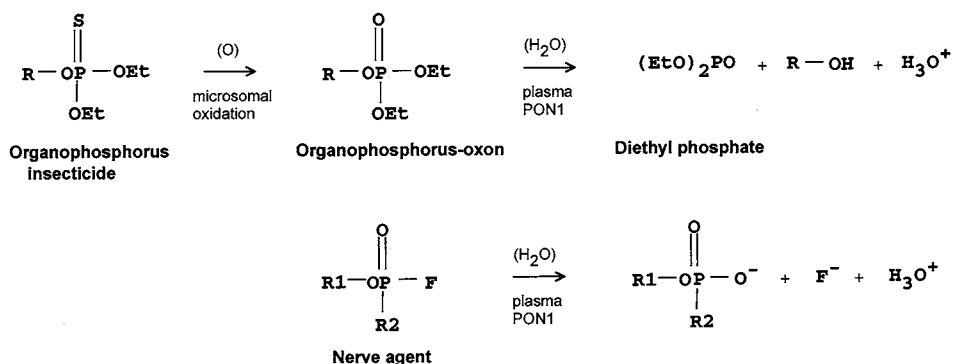

Fig. 1. The cytochrome P450/PON1 pathway for the bioactivation and subsequent detoxication of several organophosphorus insecticides and the nerve agents soman and sarin.

acute toxicity of a number of OPs. These studies represent an extension and refinement of the early enzyme injection experiment of Main [20]. In our studies, PON1 was first purified to homogeneity from rabbit serum [10]. The pure enzyme was injected in the tail vein of rats, thus elevating the hydrolyzing activity toward paraoxon and chloropyrifos oxon by nine- and 50-fold, respectively [24]. The toxicity of the two compounds was then assessed by measuring the degree of cholinesterase inhibition in plasma, red blood cells, diaphragm and brain, following exposure by the oral, dermal, i.p. or i.v. route. Animals that had been pre-treated with PON1 were consistently less sensitive to the effects of the two OPs than control rats [24].

Further studies were then carried out in mice, as this animal species requires less purified enzyme for protection experiments, and is also ideal for genetic manipulations. Initial studies in mice were devoted to investigate the degree of increase of plasma PON1 activity when the purified enzyme was given by different routes. While following i.v. injection the increase in plasma PON1 activity only lasts a few hours, we found that administration of rabbit PON1 by the i.v. + i.p. or i.v. + i.m. routes could increase serum activity in mice for more than 30 h [25]. Similar to what was observed in rats, pre-treatment with PON1 offered protection (measured by the degree of cholinesterase inhibition) against the toxicity of chlorpyrifos oxon (administered dermally, as this is one of the major routes of occupational exposure) [25]. When animals were challenged with the parent compound chlorpyrifos, utilizing a similar experimental protocol, a smaller, but still significant degree of protection was also observed, particularly in brain and diaphragm [26]. In these experiments, plasma PON1 activity was elevated by 27–34-fold by the injection of exogenous rabbit PON1 (given by the i.v. route 30 min before the OP). Since the half-life of rabbit PON1 given by the i.v. + i.p. route is very long, in an additional experiment mice were challenged with chlorpyrifos 24 h, instead of 30 min after the i.v. + i.p. injections of the enzyme. At 24 h, plasma chlorpyrifos oxonase was still 20-fold higher than controls, and mice were still protected against the acute toxicity of chlorpyrifos [26].

Additional experiments were carried out to determine whether PON1 would still be effective when administered after, rather than before, exposure to chlorpyrifos. When mice received PON1 30 min following dermal chlorpyrifos exposure, the enzyme did prevent the reduction of cholinesterase activity in all tissues [26]. When PON1 was injected 3 h after chlorpyrifos, a protective effect was seen only in brain and diaphragm cholinesterase. The dose of chlorpyrifos used in the above studies (100 mg/kg) significantly inhibited cholinesterase activity but did not cause overt signs of toxicity. Therefore, a higher dose of chlorpyrifos (150 mg/kg), which caused significant cholinergic toxicity, was selected to test the effectiveness of PON1 under conditions of severe chlorpyrifos poisoning. Mice receiving PON1 30 min after 150 mg/kg chlorpyrifos showed significant protection against cholinesterase inhibition and did not display any cholinergic signs [26].

Further experiments have investigated the toxicity of chlorpyrifos oxon and chlorpyrifos in *Pon1*-knockout mice. *Pon1* gene knockout mice were produced by targeted disruption of exon 1 of *Pon1* gene and had normal appearance and body

weights [27]. Homozygous mutant mice (*Pon1* $-/-$) had no detectable levels of PON1 protein and PON1 activity in plasma [27]. When chlorpyrifos oxon was applied dermally to *Pon1* $+/+$ and *Pon1* $-/-$ mice, the latter were significantly more sensitive to the toxicity of the OP. For example, following administration of 3 mg/kg, brain and diaphragm cholinesterase activity were inhibited by 31 and 1%, respectively, in *Pon1* $+/+$ mice, but were decreased by 88 and 91% in *Pon1* $-/-$ mice [27]. Sensitivity to chlorpyrifos also differed between wild mice and knockouts: administration of 250 mg/kg chlorpyrifos caused a 22% inhibition of brain cholinesterase in *Pon1* $+/+$ mice, but a 60% inhibition in *Pon1* $-/-$ mice [27]. On the other hand, guthion, whose metabolism does not involve hydrolysis by PON1, caused similar brain cholinesterase inhibition in both *Pon1* $+/+$ (69%) and *Pon1* $-/-$ (72%) mice (Li et al., unpublished results).

Altogether, these data provide strong evidence that PON1 is a major determinant of toxicity for those OPs whose oxons are substrates for this enzyme. An additional area in which PON1 may play a relevant role is in the developmental toxicity of OPs. It is known that the acute toxicity of OPs is influenced by age, with substantial evidence indicating that young animals are more sensitive than adults [28–32]. Intrinsic differences in brain acetylcholinesterase do not account for the age-related differences in sensitivity, as indicated by in vitro studies [30,33,34]. On the other hand, lower metabolic abilities of young animals appear to be a major determinant for their increased sensitivity to acute OP toxicity [30,35,36]. In particular, metabolic studies with parathion, methylparathion and chlorpyrifos have concluded that a lower detoxication of the respective oxons by PON1 accounts for the differential age-related sensitivity to their toxicity [30,37]. Limited evidence also exists in species other than rodents. For example, in cattle, the toxicity of chlorpyrifos is 30-fold higher in calves than in adult animals, while for disulfoton (which is not metabolized by PON1), the difference is only twofold [38].

The activity of serum PON1, measured with different substrates, increases with age in both mice and rats, with a parallel increase in hepatic mRNA levels, reaching adult levels at about postnatal week 4 [15]. There is also limited human evidence than serum PON1 activity is low in infants and children [39,40]. Furthermore, evidence from rats indicates that pregnancy is associated with reduction in serum PON1 activity [41]. Thus, these data suggest that the higher sensitivity of young animals to OP toxicity may be explained, at least in part, by a deficiency in PON1 activity.

3. Assessment of PON1 status in individuals

La Du and coworkers pioneered the development of two-dimensional assays for characterizing enzyme population distributions for PON1 [4]. Recently, we found that plotting the rates of hydrolysis of diazoxon versus paraoxon provided population frequency distributions that clearly separated individuals into the three genotypes, QQ, QR and RR (Fig. 2) [42]. In addition to genotype, this analysis provides the relative level of PON1 for each individual in the population studied. This is an
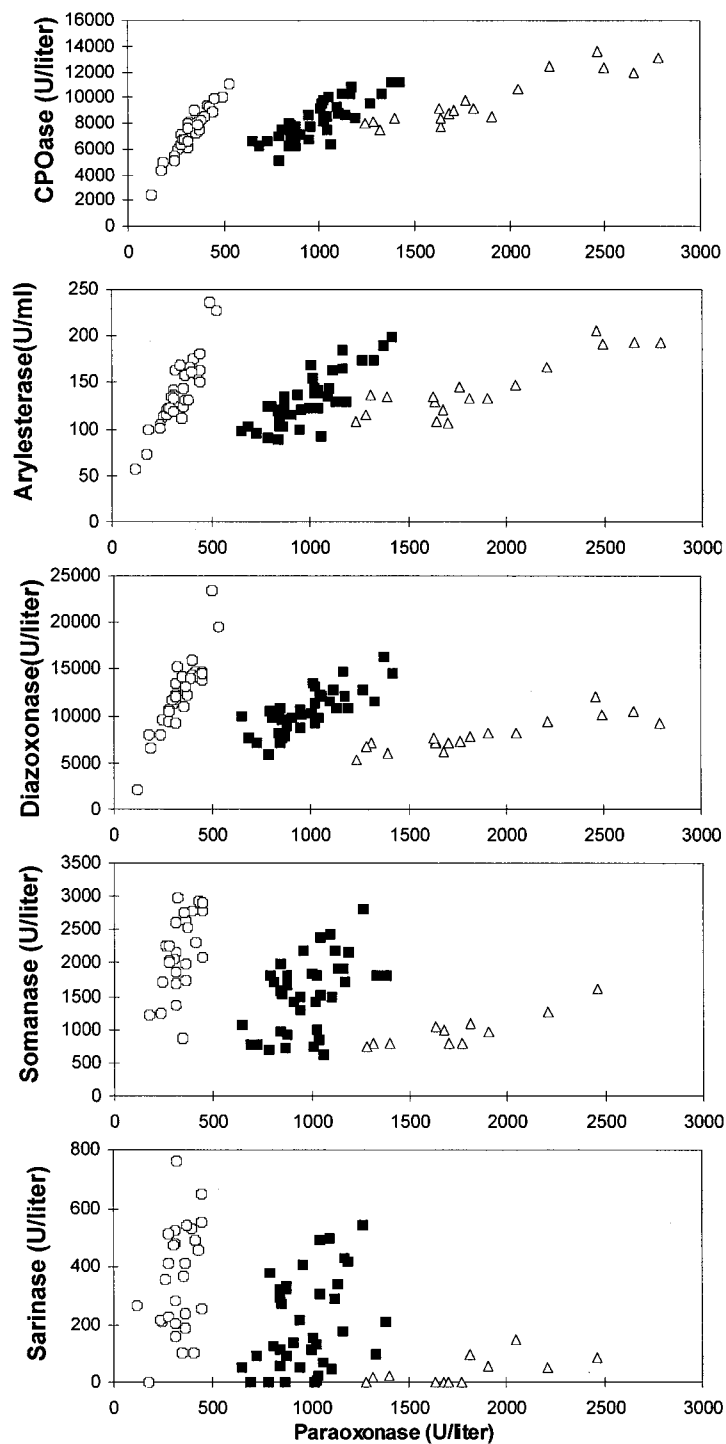
Fig. 2.

equally important 'genetic variable', since there is as much as a 15-fold variability between individuals within a given genetic class. This series of plots also shows the two-dimensional analysis for rates of hydrolysis of chlorpyrifos, phenylacetate, soman and sarin plotted against the rates of paraoxon hydrolysis for a population of Hispanic farm workers (Fig. 2).

With the advent of polymerase chain reaction amplification of DNA sequences, it has become fashionable to set up high-through-put PCR analyses to provide genotyping of populations with the aim of identifying at-risk individuals. A number of studies examining the role of the PON1 polymorphism in vascular disease have recently been reported [43–48]. All of these studies have made use of PCR analysis for genotyping subjects. It should be clear from the genetic variability observed within each genotype, that a two-dimensional analysis will provide more useful information than genotyping alone. A hypothetical example can be postulated from Fig. 2c. If individuals with rates of hydrolysis of diazoxon below 10 000 U/l would be considered at risk for a given exposure, all three genotypes would be included, but at very different percentages for each genotype (24% in QQ group; 44% in QR group; and 83% in RR group).

4. Conclusions

The animal studies carried out to date provide convincing evidence that PON1 status plays a major role in determining sensitivity or resistance to OP compounds processed through the P450/PON1 pathway. This conclusion is particularly valid for chlorpyrifos and chlorpyrifos oxon, the compounds that have been most extensively studied in the mouse model. Similar studies need to be carried out for the other OP compounds processed via the P450/PON1 pathway or compounds such as nerve agents that are hydrolyzed by PON1 directly. At the present time, the two-dimensional enzyme analysis utilizing diazoxon and paraoxon appears to provide the best determination of PON1 status. This analysis can be carried out as a high-through-put semi-automated procedure using the Molecular Devices Spectrophotometer/Plate Reader which provides kinetic analysis at variable wavelengths (Furlong and Richter, manuscript in preparation). It would be useful to identify non-toxic substrates to use for this analysis.

The data available on the developmental time course of appearance of PON1 in plasma suggest that it is very important to avoid exposure of infants to toxic OP compounds for which they may have a greatly diminished detoxication capacity. Further, in examining the population enzyme distribution plots, it is also clear that there are individuals within populations that never develop high levels of PON1 and

Fig. 2. Population distribution plots of: (a) chlorpyrifos-oxonase versus paraoxonase; (b) arylesterase versus paraoxonase; (c) diazoxonase versus paraoxonase ($n = 92$, a–c); (d) somanase versus paraoxonase ($n = 75$); and (e), sarinase versus paraoxonase ($n = 78$). ○, QQ individuals (Gln192 homozygotes); ■, QR individuals (heterozygotes); and △, RR individuals (Arg192 homozygotes). Genotype assignments were made from (c) (reproduced from Ref. [42], with permission).

may therefore be quite sensitive to the OP compounds processed via pathways involving inactivation by PON1.

Acknowledgements

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